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转人 α-乳清白蛋白基因烟草中半胱氨酸含量的提高*

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摘要: 将人 α-乳清白蛋白基因构建到植物表达载体上, 通过农杆菌介导采用叶盘法将人 α-乳清白蛋白基因导入到烟草中, 经过 PCR 和 Southern blot 分析表明: 人 α-乳清白蛋白基因已经整合到烟草基因组中; 经过 RT-PCR 和 GUS 组织化学染色证明: 人 α-乳清白蛋白基因得到了表达。测定了 9 株转基因烟草叶片半胱氨酸含量, 大部分植株有着明显的提高, 最高幅度达到了 318.02%, 半胱氨酸含量平均提高了 166.40%。
关键词: 人 α-乳清白蛋白基因; 转基因烟草; 半胱氨酸; 提高
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Transformation of Human Alpha-lactalbumin Gene into Tobacco and Improvement of Cysteine Contents in Transgenic Tobacco Plants

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Abstract: The plant expression vector containing human alpha-lactalbumin gene was constructed and its gene was transformed into tobacco using leaf disc mediated by *Agrobacterium tumefaciens* LBA 4404 as a vehicle. PCR and Southern blot analyses showed that human alpha-lactalbumin gene was integrated into the tobacco genome. RT-PCR and histochemical staining for GUS activity demonstrated that human alpha-lactalbumin gene was expressed in transgenic tobacco plants. Further analysis of the data indicated that the average of cysteine contents in 9 transgenic tobacco plants was greatly increased by 166.40% besides one of them which was increased by 318.02%.
Key words: Human alpha-lactalbumin gene; Transgenic tobacco plants; Cysteine; Improvement

Human alpha-lactalbumin (α-LA) is a small, acidic, Ca^{2+} binding protein, secreted in the female mammary gland (Wim *et al.*, 1999; Yu *et al.*, 2004). α-LA content is higher in human milk with about 28 percent of the total protein. It contains rich tryptophan and cysteine. Tryptophan is one of the essential amino acids which can not be synthesize by human body itself. Cysteine is the composition of glutathione and can protect cells from being harmed by oxidation. More importantly, α-LA plays an important role in physiological and health functions, for instance, the resistance to the most Gram-positive bacteria, killing the pneumococcus, particularly, inducing apoptosis in tumor cells (Walter *et al.*, 2004; Hakansson *et al.*, 2000; Svensson *et al.*, 1999; Hakansson *et al.*, 1995; Kohler *et al.*, 1999; Pettersson *et al.*, 2006; Gustafsson *et al.*, 2005). The α-LA genomic DNA has been transformed into the rats by means of genetic engi-

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neering and it has been expressed in transgenic rat (Yu *et al.*, 2004; Liu *et al.*, 2004; Fujiwara *et al.*, 1997; Fujiwara *et al.*, 1999; Fujiwara *et al.*, 2003).

In comparison with the animal expression system, the plant expression system has many advantages with easy operation, planting in field in large scale, low cost and expressed protein stored in seeds etc (Wang *et al.*, 2002). Thus, ones pay much more attention to utilizing plants as the biological reactor to produce the proteins which human beings need. With the continuous improvement of Chinese people's food quality and their living standards, the requirements for food nutrition are being raised daily (Gao *et al.*, 2001). This paper presents our work on the construction of the plant expression vector pBRALA, the expression of human α-LA gene in transgenic tobacco plants, and the determination of the cysteine content of transgenic tobacco plants. The results could lay down a foundation for studying the production and utilization of human α-LA using the method of plant genetic engineering.

1 Materials and Methods

1.1 Materials

1.1.1 Plant materials Wild type tobacco Yunyan 85 was kept in our laboratory.

1.1.2 Strains and plamids *Escherichia coli* DH5α, *Agrobacterium tumefaciens* LBA4404, plasmids of pCAMBIA-1301, pSPROK and pHALA were stored in our laboratory. The plamid pHALA contains 375 bp fragment encoded for human alpha-lactalbumin. Plasmid pBRALA was constructed for this experiment.

1.1.3 Main chemicals Restriction enzymes, T4-DNA ligase, Taq DNA polymerase, Takara RNAiso Reagent, PrimeScript™ RT-PCR Kit etc were purchased from the Takara Biotechnology (Dalian) Co., Ltd; IPTG, X-gal, X-gluc and DNA Purification and Recovery Kit were obtained from the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd; and Hygromycin B and DIG High Prime DNA Labeling and Detection Starter Kit II were used from the Roche Diagnostics Corporation; DNA marker II was bought from the Tiangen biotech (Beijing) Co., Ltd.

1.1.4 PCR primers According to a part of DNA sequence of human α-LA gene, a pair of the primers were designed: 5′ primer was 5′-CAATGAAAGCACGGAATA-3′ and 3′ primer was 5′-AACTTCTC ACAAAGCCAC-3′. The length of DNA sequence between the pairs of primers was 237 bp. The primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.

1.2 Methods

1.2.1 Construction of the plant expression vector Two fragments, one from pCAMBIA-1301 and the other one from pSPROK, containing CaMV 35S promoter and nos terminator after restriction with *EcoR* I/*Hind* III and recovery, were ligated with T4-DNA ligase. Then it was transformed into the competent cells of *Escherichia coli* DH5α, and the *E. coli* DH5α was spread onto LB medium containing kanamycin, IPTG and X-gal. White colonies were screened and the recombinant plasmid pCBROK was obtained (Zhang *et al.*, 2000; Gao *et al.*, 2001). The HALA fragment recovered from plasmid pHALA, restricted with *BamH* I/*Kpn* I, was inserted into the plasmid pCBROK cut by *BamH* I/*Kpn* I to obtain the plant expression vector pBRALA.

1.2.2 Transformation of plant expression vector pBRALA into *Agrobacterium tumefaciens* LBA4404 The plant expression vector pBRALA was introduced into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method (Hofen *et al.*, 1988).

1.2.3 Preparation of axenic tobacco seedlings Tobacco seeds were surface-sterilized in 70% ethanol for 1 min, rinsed three times in sterile distilled water, and then were in 0.1% silver nitrate for 5 min, washed three times with sterile distilled water. The surface-sterilized tobacco seeds were planted on half-strength MS medium solidified with 0.75% agar for germination to obtain seedlings. The seedlings were grown in a growth chamber with a photoperiod of 16 h light/8 h of darkness at a temperature of 25℃ (Nancy and Maud, 1994).

1.2.4 Transformation of tobacco When the seedlings were 4-6 weeks old, the young leaves were harvested and cut into small pieces of leaf discs. Then they were immersed in the *Agrobacterium tumefaciens* cell solution for 15 min, dried using sterile filter paper to remove excessive bacteria solution and placed on co-culture medium without antibiotics. After co-cultured in the dark for 48 h at 25℃, They were placed on the selection medium. When the buds with 1-2 cm in length appeared on the selection medium, they were removed carefully from the explants and transferred into the rooting medium. After tobacco shoots had rooted, the plantlets were transferred to the pots containing watered soil. The pots with the plantlets were covered with a plastic bag for 1 week in a growth chamber in order to keep the humidity at a relatively high level. Then the plantlets were grown in a growth chamber with a photoperiod of 16 h light/8 h of darkness at a temperature of 25℃ and if necessary, they were watered again. The media for transformation of tobacco were as follows: (1) The co-culture medium: MS basal medium + 0.5 mg/L 6-BA + 0.1 mg/L IAA; (2) The selection medium: MS basal medium + 0.5 mg/L 6-BA + 0.1 mg/L IAA + 300 mg/L cefotaxime + 20 mg/L Hygromycin B; and (3) The rooting me-

dium: MS basal medium + 0.1 mg/L IAA + 200 mg/L cefotaxime + 10 mg/L Hygromycin B (Nancy and Maud, 1994).

1.2.5 PCR analysis Isolation of plant genomic DNA was performed according to Roger *et al.* (1988). The PCR amplification was made by using the genomic DNA from transformed tobacco plants as templates according to Sambrook *et al.* The PCR reaction condition was: after 94℃ for 5 min, 94℃ for 1 min, 50℃ for 1 min, 72℃ for 1 min, 35 cycles, 72℃ for 10 min at the end.

1.2.6 Southern blotting analysis Analysis of Southern blotting for transformed tobacco plants was conducted according to Sambrook *et al*. The tobacco genomic DNA, after being restricted with *BamH* I/*Kpn* I completely and separated on an agarose gel, was transferred into the positive charged nylon membranes by a capillary transfer method. DIG-labeled DNA probe, hybridization and detection were performed according to the instruction manual of DIG High Prime DNA Labeling and Detection Starter Kit II (Zhang *et al.*, 2000; Gao *et al.*, 2001).

1.2.7 RT-PCR analysis All the vessels for isolation of RNA were treated to be RNase-free. The glass and metal wares wrapped in aluminum foils and baked at 180℃ for 12 h. The plastic wares, such as eppendorf tubes and pipette tips, were soaked in freshly prepared 0.1% (V/V) diethylpyrocarborate (DEPC) water for 16 h at 37℃ and then autoclaved for 30min at 121℃. Isolation of total RNAs from the transgenic tobacco leaves was conducted according to the instruction manual of Takara RNAiso Reagent. Analysis of RNA quality was performed by electrophoresis through 1.2% agarose gel, stained with ethidium bromide to see three bands of rRNA. RT-PCR was carried out according to the instruction manual of PrimeScript™ RT-PCR Kit.

1.2.8 Histochemical staining of GUS activity Histochemical staining of GUS activity was carried out according to Jefferson *et al.* (1987). The leaves of transformed tobacco plants and their roots were soaked in GUS reaction mixture containing 2 mmol L X-Gluc and incubated at 37℃ for 12 h. After staining, the green color of the leaves was decolorized by ethanol and the roots were made transparent by 12% sodium hypochlorite for 40 seconds after they were rinsed three times in sterile distilled water. Free-hand sections of roots with stained blue were made and observed under a light microscope (Zhang *et al.*, 2000; Gao *et al.*, 2001).

1.2.9 Determination of amino acid content of transgenic tobacco plants About 20 grams of leaves from each transgenic tobacco plant were taken and the content of amino acids was determined at the Institute of Subtropical Forestry, the Chinese Academy of Forestry Sciences. The data obtained from each transgenic tobacco plant were analyzed according to the following formulas:

formula 1: $A = B \div C$;

formula 2: $D = (A_i - A_{ck}) \div A_{ck}$.

where A is the percentage of the cysteine in total amino acids in each sample, B is the cysteine contents of the sample, C is the total amino acid content of the sample, D is the percentage of the cysteine improved in transgenic plants, A_i is the percentage of the cysteine in each transgenic plant, A_{ck}. is the percentage of the cysteine in non-transformed plants as the control, and i is used in each transgenic plant, while ck is the non-transformed plants as the control.

2 Results

2.1 Vector construction

The multiple cloning site (MCS) of plant expression vector pCAMBIA-1301 are inside *lacZ* gene. After insertion of the gene into MCS, the *lacZ* gene is inactivated, resulting in obtaining recombinants according to white colonies. The fragment of P35s-Tnos from plasmid pSPROK restricted with *EcoR* I/*Hind* III was inserted into the MCS to obtain the recombinants named pCBROK. The HALA gene in length of 375 bp from plasmid pHALA cut by *BamH* I/*Kpn* I was integrated into the corresponding site of plasmid pCBROK to obtain the plant expression vector pBRALA, which was suitable to make the transformation of dicotyls (such as tobacco) via *Agrobacterium tumefaciens*. The procedure for vector construction and the restriction endonuclease map were shown in Fig. 1 and Fig. 2 respectively.

2.2 Production of transgenic tobacco plants and their molecular identifications

2.2.1 Transformation of vector pBRALA into tobacco via *Agrobacterium tumefaciens* The young tobacco leaves were selected as explants for the transformation when the axenic tobacco seedlings were 30 - 40 days old. After infected with *Agrobacterium tumefaciens* and co-cultured, the explants were grown in the selection medium for plant regeneration. After 30 days later, the buds appeared and were cultured continuously till tobacco with shoots and roots formed. After the rooted plantlets were further developed, they were transplanted to the large pots until blooming and the production of seeds. Finally, the 38 transformed tobacco plantlets were produced for this experiment.

2.2.2 PCR analysis of the transformed tobacco plants The genomic DNA isolated from the leaves of transgenic tobacco plants was taken as the PCR template

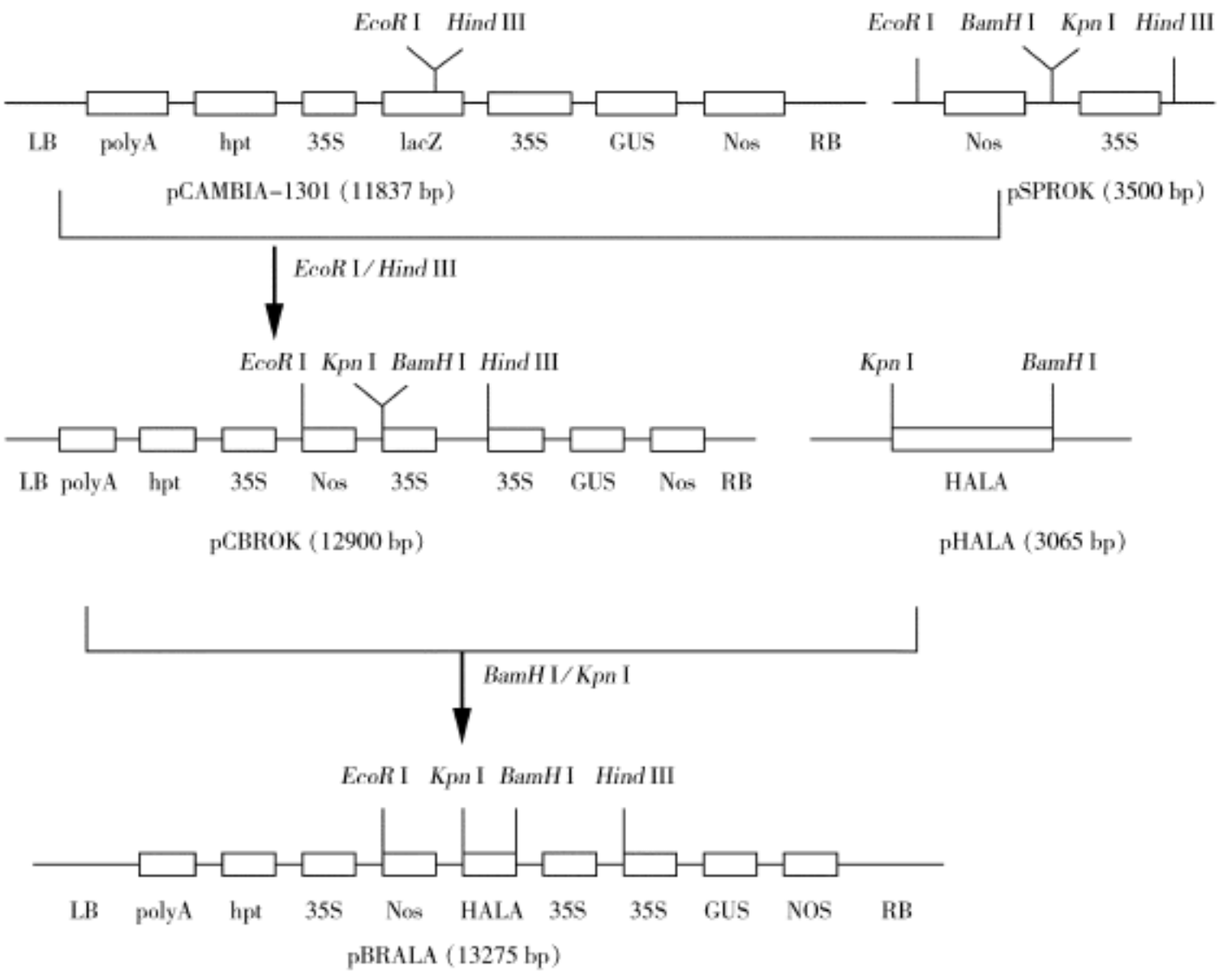

Fig . 1 Construction of plant expression vector pBRALA

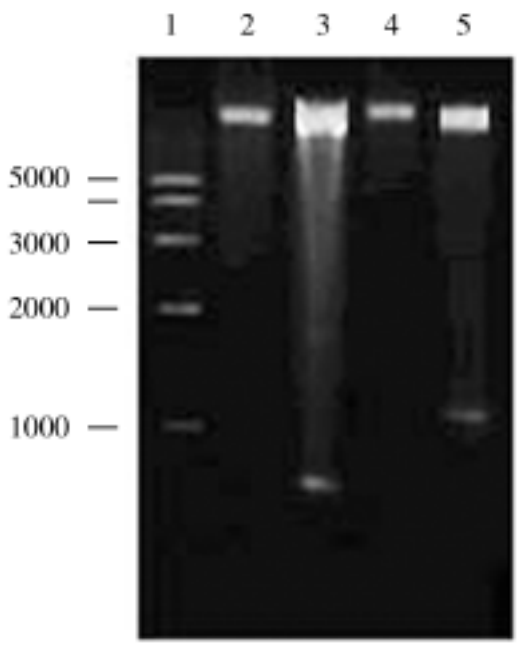

Fig . 2 Restriction enzyme analysis of plant expression plasmid pBRALA

Lane 1, DNA molecular marker (1 kb DNA Ladder); Lane 2, plasmid pBRALA; Lane 3, pBRALA/*BamH* I + *Kpn* I; Lane 4, plasmid pCBROK; Lane 5, pCBROK/*Hind* III + *EcoR* I

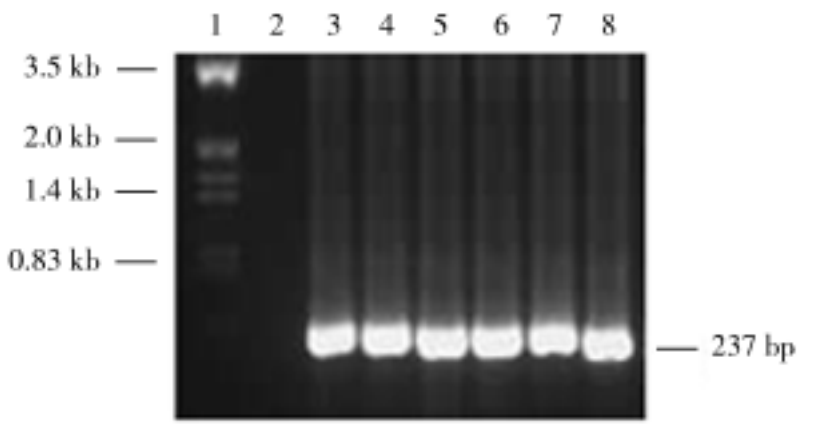

Fig . 3 PCR analysis of transformed tobacco plants

Lane 1, DNA molecular marker (λ DNA/*Hind* III + *EcoR* I); Lane 2, non-transformed tobacco plant DNA as the negative control; Lane 3, plasmid pBRALA as the positive control; Lane 4-8, the amplified products from transformed tobacco plants

and the synthetic oligodeoxyribonucleotides as primers were taken together to do PCR . The 15 μl PCR product was taken to run electrophoresis of 1.2% agarose gel . The result was shown in Fig . 3 . The amplified product indicated that the size of the specific band with 0.2 kb from the transgenic tobacco plants was the same as the positive control, but the non-transformed tobacco plants as the negative control did not show any amplified bands .

2.2.3 Southern blotting analysis of the transformed tobacco plants The Southern blotting analysis of DNA from some of transformed tobacco plants was made using the HALA gene as a probe labeled with DIG-dUTP and the result was shown in Fig. 4. It is indicated that the HALA gene had integrated into the genome of the transformed tobacco plants.

2.2.4 RT-PCR analysis of the transformed tobacco plants The RT-PCR analysis for some transgenic tobacco plants with positive southern blotting was performed and the result was shown in Fig. 5. It is indicated that the HALA gene was expressed at the RNA level.

2.2.5 Expression of gus gene in transgenic tobacco plants The transformed tobacco leaves and young roots were placed into a GUS reaction mixture. The results of GUS staining were shown in Fig. 6. It is demonstrated that GUS activity in transformed tobacco leaves and young roots was expressed, but in non-transformed tobacco materials as the control was not expressed.

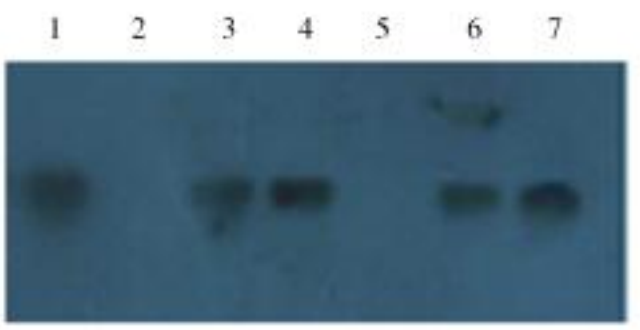

Fig. 4 Southern blot analysis of transgenic tobacco plants
1, plasmid pBRALA/*BamH* I + *Kpn* I as the positive control; 2, 5, genomic DNA of non-transformed tobacco plants digested with *BamH* I + *Kpn* I as the negative control; 3, 4, 6, 7, genomic DNA of transformed tobacco plants digested with *BamH* I + *Kpn* I

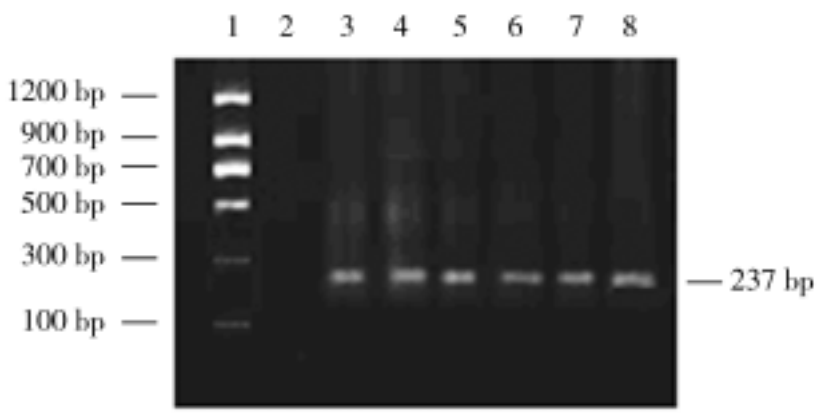

Fig. 5 RT-PCR analysis for transgenic tobacco plants
Lane 1, DNA molecular marker (DNA marker II); Lane 2, non-transformed tobacco plant as the negative control; Lane 3-8, the amplified products from transformed tobacco plants

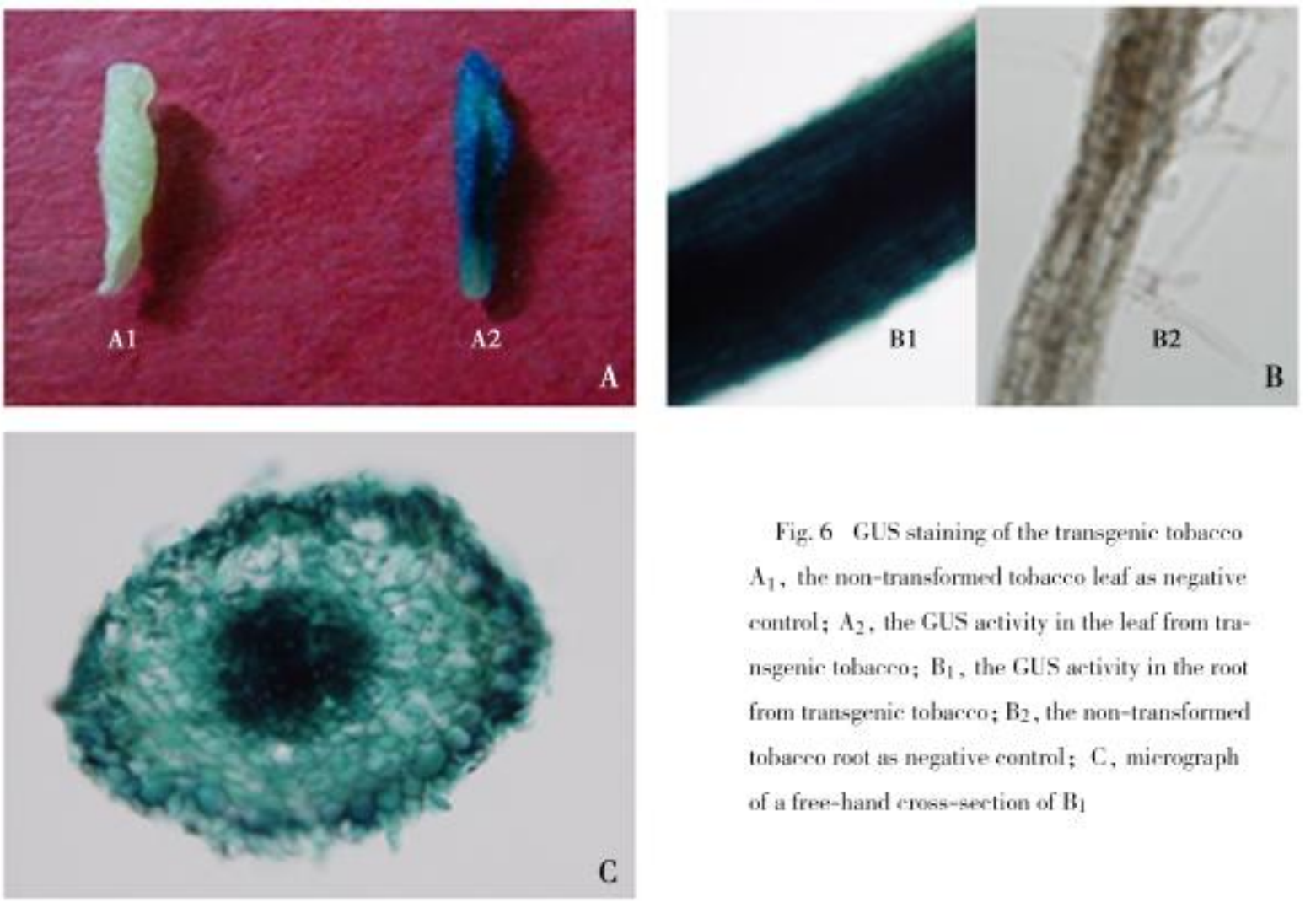

Fig. 6 GUS staining of the transgenic tobacco
A_1, the non-transformed tobacco leaf as negative control; A_2, the GUS activity in the leaf from transgenic tobacco; B_1, the GUS activity in the root from transgenic tobacco; B_2, the non-transformed tobacco root as negative control; C, micrograph of a free-hand cross-section of B_1

Table 1 The cysteine percentage and improving percentage in each measured samples

	Measured tobacco leaves									
	ck	1	2	3	4	5	6	7	8	9
Cysteine percentage in total amino acids (%)	1.11	2.72	0.55	1.90	2.13	3.61	4.64	4.30	4.00	2.76
Improving percentage of cysteine (%)	0	145.05	-50.45	72.07	91.89	225.23	318.02	287.39	260.36	148.65

where ck was the leaves in non-transformed plants as the control, 1, 2, 3…… were the leaves in transgenic plants

2.2.6 Cysteine measurement of transgenic tobacco plants The cysteine contents in leaves from 9 transgenic plants with positive reactions of PCR, Southern blotting, RT-PCR and GUS staining were determined. The cysteine percentage and improved percentage in each measured sample was shown in Table 1.

Table 1 indicated that most of the transgenic tobacco plants obviously contain more cysteine contents than the control. Among them, 6 transgenic plants showed their cysteine contents were improved by more than 140%, and one of them was increased by 318.02%. Data analysis showed that the average of the improved percentage of cysteine was 166.40%.

3 Discussions

3.1 Production of the protein in transgenic plants

It was reported that the proteins or amino acids which human beings needs were produced by transgenic plants. The human beta-casein gene was transformed into potato and expressed in transgenic potato (Chong *et al.*, 1997). The soybean ferritin gene was transferred into rice and the contents of ferritin were increased by three-folds in seeds of T1 transgenic rice plants. Thus, the contents of iron in transgenic rice were improved. If people had such transgenic rice, the iron would be taken into the bodies (Goto *et al.*, 1999). The gene encoded for respiratory syncytial virus-F protein was introduced into tomato plants and transgenic tomato plants were produced. The mice had transgenic tomatoes, their systemic immune response to respiratory syncytial virus was induced and the specific antibody was produced. These results indicated that the new method of the production of oral respiratory syncytial virus-F vaccine was developed (Jagdeep *et al.*, 2000). The lysine-rich protein gene cloned from *Psophocarpus tetragonolobus*, was transferred into rice and lysine content in most of the transgenic plants, which were obviously improved, and one of them increased by 16.04% (Gao *et al.*, 2001). These articles suggested that the proteins and amino acids which human beings needs were produced via the plant expressed system, and the ingredients for health were improved, thus, the high quality of the plant food also could be improve upon. In addition, it was significant that the genes encoded special proteins rooted from animals or bacteria were introduced into the plants and the expressed systems of production of these proteins were enlarged. In this paper, the human alpha-lactalbumin gene was transformed into tobacco in order to make some reference to research into the production and utilization of human α-LA.

3.2 The detection of the human alpha-lactalbumin in transgenic tobacco

In this paper, we carried out PCR analysis and Southern blotting, but did not do Western blotting. We will do it in future in order to study the expression of the human alpha-lactalbumin gene at the protein level. It is important to investigate the expression and regulation of the integrated gene at protein level.

3.3 The function of extrinsic proteins in transgenic tobacco plants

In this paper, transformation of the human alpha-lactalbumin gene into tobacco was made and transgenic tobacco plants were obtained, it is not enough. In future, the function of the human alpha-lactalbumin expressed in transgenic tobacco plants would be further studied, and what is the difference between the proteins synthesized in two expressed systems, i.e., animal expressed system and plant expressed system, would be known and explored.

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